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Distribution and Clearance of Aflatoxins B_1 and M_1 in Turkeys Fed Diets Containing 50 or 150 ppb Aflatoxin from Naturally Contaminated Corn

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SUMMARY. Turkeys were fed a diet containing 50 or 150 ppb aflatoxin for 11 or 13 weeks or fed these diets for 11 weeks and then the control diet for 1 or 2 weeks. Aflatoxins B_1 and M_1 were found in liver, kidney, gizzard, and feces of poults fed the diets for 11 or 13 weeks. However, in turkeys fed the control diet for 1 or 2 weeks after the 11-week feeding trial, no residues of aflatoxin were found in the feces or tissues, except for some aflatoxin B_1 remaining in detectable amounts in the gizzard. No mortality was attributable to aflatoxin, and there were no notable differences among groups in weight gains, feed conversion, or histopathologic changes in selected tissues. The response to a second inoculation with sheep erythrocytes was significantly lower in poults given dietary aflatoxin than in controls. This reduced antibody response was not observed when a *Pasteurella multocida* vaccine was administered.

RESUMEN. Distribución y eliminación de aflatoxinas B_1 y M_1 en pavos alimentados con dietas que contenían 50 o 150 ppb de aflatoxina procedente de maíz contaminado en forma natural.

Se alimentaron pavos con una dieta que contenia 50 o 150 ppb de aflatoxina durante 11 o 13 semanas, o durante 11 semanas y luego una dieta control durante 1 o 2 semanas. Se encontraron aflatoxinas B_1 y M_1 en el hígado, riñón, molleja y heces de pavitos alimentados con las dietas de 11 o 13 semanas. Sin embargo, no se detectaron aflatoxinas en las heces o tejidos en pavos alimentados con la dieta control de 1 o 2 semanas después de la dieta con aflatoxinas, con excepción de cierta cantidad detectable en la molleja. No se atribuyó mortalidad alguna a las aflatoxinas y no hubo diferencias notables entre los grupos en cuanto a ganancia de peso, conversión de alimento o cambios histopatológicos en algunos tejidos. La respuesta a una segunda inoculación con eritrocitos de oveja se vió disminuída en pavos alimentados con la dieta con aflatoxinas, en comparación con los controles. Sin embargo, no se observó esta reducción en la respuesta humoral cuando se utilizó una vacuna de *Pasteurella multocida*.

The analysis of aflatoxin residues in tissues of poultry following ingestion of various concentrations of dietary aflatoxins has been the subject of several reports (2,3,8,11,12). When nine laying hens consumed 8 ppm dietary aflatoxin B_1 (AFB_1) for 7 days, AFB_1 was found in the liver of one of the hens after a 7-day withdrawal period (12). Gregory *et al.* (2) found that 55–91% of the detectable aflatoxin residues in turkey poults fed 500 ppb dietary AFB_1 were conjugated aflatoxins rather than AFB_1 or the metabolite aflatoxin M_1 (AFM_1). Their study was conducted using dietary concentrations of aflatoxin that caused clinical aflatoxicosis in the poults. However, AFB_1 or AFM_1 could be found in several tissues and body fluids of steers fed concentrations of dietary aflatoxins that were less than needed to cause aflatoxicosis (5).

Therefore, we conducted the present study to determine the presence of AFB_1 and AFM_1 in tissues of turkey poults fed low concentrations (50 and 150 ppb) of AFB_1 in the diet and to determine the disappearance of the residues following withdrawal of the contaminated diets from the poults. Additionally, we wanted to determine the effect of these dietary concentrations of aflatoxin on weight gains, antibody formation to *Pasteurella multocida* and sheep erythrocytes, feed conversion, liver-to-body-weight ratios, and histopathologic changes of selected tissues.

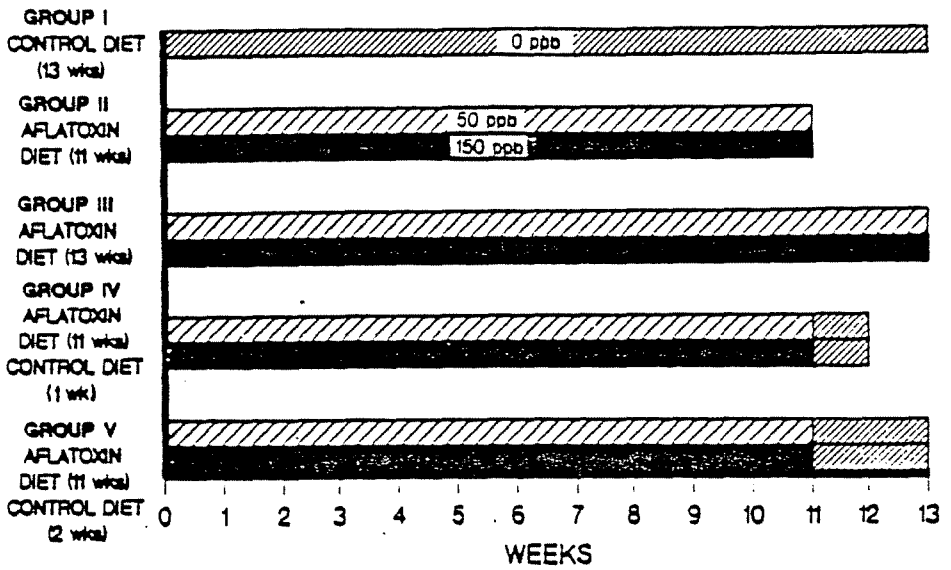

Fig. 1. Experimental design of groups of turkeys fed 0, 50, or 150 ppb dietary aflatoxin for 11 or 13 weeks followed by withdrawal periods of 1 or 2 weeks (Groups IV and V, respectively).

MATERIALS AND METHODS

Turkeys. Turkey poults (n = 110) were obtained at 1 day of age and reared at the National Animal Disease Center until they were 2 weeks old. They were given water and turkey starter (assayed to be free of aflatoxin) *ad libitum*.

Feed. Three batches of feed were prepared: one contained 0 ppb AFB_1, another contained 50 ppb AFB_1, and the third contained 150 ppb AFB_1. The feed was prepared by mixing naturally contaminated ground corn (assayed for aflatoxin concentration in two laboratories) with turkey starter to yield the desired aflatoxin concentration in the final feed mixture. Corn assayed to be free of aflatoxin was included in the 50-ppb ration and the control ration to maintain an equivalent amount of corn in all rations. All rations were stored at 4 C during the study.

Experimental design. The two-week-old poults were randomly assigned to five groups as shown in Fig. 1. Thirty poults were assigned to Group I and were fed the control ration. Twenty poults were assigned to each of the other four groups; 10 poults in each group were fed 50 ppb AFB_1 ration, and the remaining 10 per group were fed 150 ppb AFB_1 ration (Fig. 1). The poults were housed in heated rooms with controlled ventilation on ground-corn-cob litter and given feed and water *ad libitum*.

All feed was weighed when placed in the feeders. At the end of each week, the feed remaining in each feeder was weighed so that the amount of feed consumed by each group could be determined for each week. All poults were weighed weekly, and feed conversions were determined.

Poults in Groups I and III were fed their diets for 13 weeks, and Group II poults were fed their diets for 11 weeks. Group IV poults were fed their diets for 11 weeks and then the control diet for 1 week; Group V was treated the same, except the control diet was given for 2 weeks. At the end of these periods, all poults from Groups II–V were bled, weighed, and killed. Ten control (Group I) poults were bled, weighed, and killed at each of these times also.

The liver was removed from all poults and weighed for determination of liver-to-body-weight ratios. Samples of the following were taken for aflatoxin assays: bile, blood, feces, liver, spleen, lungs, heart, kidney, gizzard, breast muscle, thigh muscle, and pancreas. All tissues were frozen at −70 C until assayed.

Tissues taken for histopathologic examination were liver, kidney, thymus, and bursa of Fabricius. All tissues were fixed in 10% neutral buffered formalin, embedded, sectioned, stained with hematoxylin and eosin, and examined with the light microscope.

Assay of tissues and fluids for AFB_1 and AFM_1. Ten-ml samples of blood from each of five poults per group were pooled by group for each assay of aflatoxin. The 50 ml of pooled blood was poured onto a 35-g hydrophilic matrix column (CT-2050 Chemtube, CT-001 Hydromatrix, Analytichem International, Inc., Harbor City, Calif.) and eluted three times with 50 ml 4:1 (v/v) methylene chloride : acetone. The pooled extracts were dried under vacuum, redissolved in methylene chloride, transferred quantitatively to dram vials, and dried under flowing nitrogen. Aflatoxins in bile were determined similarly, except the total amount of bile available for assay varied, depending upon the pooled yield from 8–10 poults. Feces and all tissue samples were analyzed individually by the method of Stubblefield and Shotwell (9). All sample extract resi-

Table 1. AFB_1 and AFM_1 in liver, kidney, gizzard, and feces of turkeys fed dietary aflatoxin for 11 or 13 weeks or fed dietary aflatoxin for 11 weeks and then a control diet for 1 or 2 weeks.

Group	Diet	Dietary aflatoxin	Liver		Kidney		Gizzard		Feces	
			B_1	M_1	B_1	M_1	B_1	M_1	B_1	M_1
I	Control		0[A]	0	0	0	0	0	0	0
II	Contaminated 11 weeks	50 ppb	7/9[B] (0.02–0.09)[C]	0	6/9 (0.01–0.02)	4/9 (0.01–0.02)	5/9 (0.043–0.162)	0	8/9 (0.08–0.45)	9/9 (0.63–1.87)
		150 ppb	7/9 (0.08–0.13)	7/9 (0.03–0.10)	4/9 (0.025–0.08)	2/9 (0.09–0.13)	6/9 (tr.–0.122)[D]	0	8/8 (0.53–4.73)	8/8 (0.35–1.7)
III	Contaminated 13 weeks	50 ppb	7/9 (0.02–0.13)	3/9 (0.11–0.14)	5/9 (0.01–0.34)	2/9 (0.01–0.07)	6/9 (tr.–0.113)	0	8/9 (0.19–0.44)	7/9 (1.16–4.54)
		150 ppb	9/9 (0.08–0.39)	9/9 (0.04–0.32)	6/9 (0.05–0.18)	1/9 (0.13)	9/9 (tr.–0.22)	2/9 (tr.)	9/9 (0.92–4.55)	9/9 (1.5–5.4)
IV	Contaminated 11 weeks, 1 week withdrawal	50 ppb	0	0	0	0	9/10 (0.037–1.9)	0	0	0
		150 ppb	0	0	0	0	9/9 (0.093–0.24)	0	0	0
V	Contaminated 11 weeks, 2 weeks withdrawal	50 ppb	0	0	0	0	5/10 (tr.–0.154)	0	0	0
		150 ppb	0	0	1/9 (0.01)	1/9 (0.04)	2/9 (0.04–0.064)	0	0	0

[A]0 = not detected.
[B]Number of tissues positive/number of tissues examined.
[C]Range of toxin concentration in ng/g.
[D]tr. = trace (<0.01 ng/g).

dues were dissolved in 100 µl 9:1 (v/v) benzene : acetonitrile for thin-layer chromatography.

Two-dimensional thin-layer chromatography and fluorometric densitometry was conducted according to the method of Stubblefield and Shotwell (9). All positive samples were confirmed with trifluoroacetic acid treatment. The limit of detection of AFB_1 and AFM_1 with this method is ≤0.1 ng/g (9).

Inoculation. All poults were inoculated with *P. multocida* vaccine and sheep red blood cells (SRBC) at 1 month and again at 2 months to determine possible interference of aflatoxin with antibody formation. The *P. multocida* vaccine was prepared from strain P-1059. An 18-hr culture grown on dextrose starch agar was harvested in phosphate-buffered saline (pH 7.4) containing 0.3% formaldehyde. The cell suspension was adjusted to a turbidity equivalent to a McFarland #10 nephelometer standard and emulsified with an equal volume of Freund's incomplete adjuvant. The SRBC inoculum was a 7% suspension of cells in 0.15 M saline (10). One ml of *Pasteurella* vaccine was inoculated subcutaneously in the cervical area, and 1 ml of the SRBC inoculum was given intravenously in the wing vein. All poults were bled before each inoculation and 2 weeks after each inoculation. Sera were assessed for antibody by an indirect *Pasteurella* hemagglutination test and by agglutination of SRBC.

Lipopolysaccharide (LPS) of *P. multocida* strain P-1059 was purified as described (7). Turkey erythrocytes were sensitized with heat-treated LPS as described (6). Passive hemagglutination tests were done with a microtiter system (Cooke Engineering Co., Warrenton, Virginia). Serial twofold dilutions of sera were made in 0.05 ml phosphate-buffered saline containing 0.5% bovine serum albumin, and 0.05 ml of LPS-sensitized turkey erythrocytes was added to each serum dilution. The plates were incubated for 1.5 hr at room temperature before passive hemagglutination reactions were assessed.

RESULTS

As expected, there were no deaths attributable to aflatoxin, and all turkeys appeared healthy throughout.

At postmortem examination, none of the poults had aflatoxin in the bile, blood, spleen, heart, lungs, breast muscle, thigh muscle, or pancreas. Furthermore, no aflatoxins were found in any blood samples, except in one sample of pooled blood from five poults given 50 ppb dietary aflatoxin that had 0.4 ng AFB_1/ml.

AFB_1 was detected in the livers of most of the turkeys given either 50 or 150 ppb dietary aflatoxin for 11 or 13 weeks (Groups II and III; Table 1). However, no AFM_1 was detected in poults given 50 ppb dietary aflatoxin for 11 weeks, and

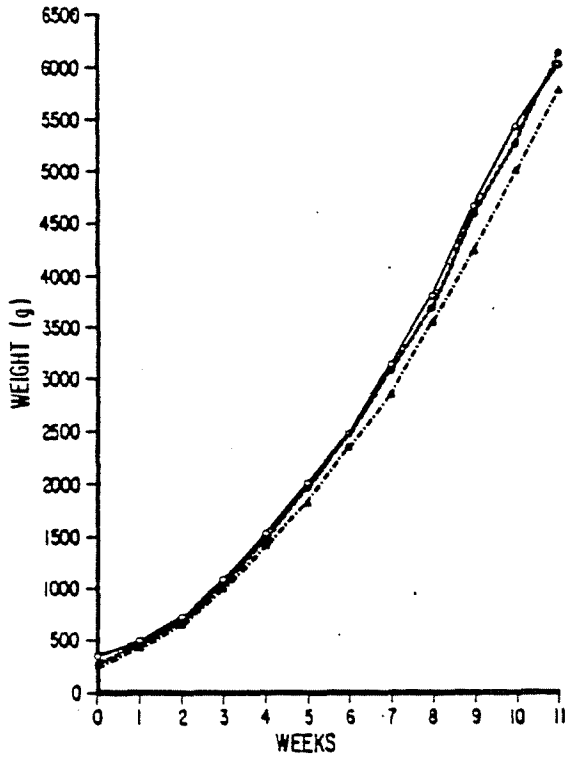

Fig. 2. Average weight gains of groups of turkeys fed 0 (○——○), 50 (●– – –●), or 150 (▲–·–·–▲) ppb dietary aflatoxin for 11 weeks.

it was detected in only 3 of 9 poults given this diet for 13 weeks. AFM_1 was found in most of the livers of poults given 150 ppb dietary aflatoxin for 11 or 13 weeeks.

Both AFB_1 and AFM_1 were found in the kidneys of many of the poults in Groups II and III, but AFM_1 was found less frequently (Table 1).

In both Groups II and III, both AFB_1 and AFM_1 occurred in the highest concentrations and in the greatest frequency in feces (Table 1).

Interestingly, AFM_1 was not detected in any of the gizzards, except in 2 of the 9 poults given 150 ppb dietary aflatoxin for 13 weeks, and then only trace amounts were detected (Table 1). AFB_1 was found in most of the gizzards of poults in both Groups II and III.

None of the turkeys fed the control diet during a 1- or 2-week withdrawal period (Groups IV and V) had detectable aflatoxins in the liver, kidney, or feces, except for one turkey fed 150 ppm dietary aflatoxin and the control diet for 2 weeks, which had AFB_1 and AFM_1 in one kidney. However, detectable AFB_1 remained in the gizzards of almost all poults after the 1-week withdrawal period and less frequently in the poults after the 2-week withdrawal period (Table 1).

Table 2. Average passive hemagglutination titer $\log_2$ of antibodies to *Pasteurella multocida* lipopolysaccharide from turkeys fed two concentrations of dietary aflatoxin and inoculated with *P. multocida* vaccine.

Dietary aflatoxin	Week 4 (before inoculum dose #1)	Week 6	Week 8 (before inoculum dose #2)	Week 10
Control	0	6.9	3.9	4.5
50 ppb	0	6.8	3.9	5.1
150 ppb	0	8.8	3.9	4.4

There were no notable differences in weight gains among the poults given 0, 50, or 150 ppb dietary aflatoxin for 11 weeks, although the poults given 150 ppb dietary aflatoxin had an average weight slightly less than poults in the other two groups beginning at week 6 of the experimental period (Fig. 2). Also, no change in feed conversion was noted among the groups.

Following the first vaccination, poults given 150 ppb dietary aflatoxin had a greater increase in antibody titer to *P. multocida* lipopolysaccharide than controls and poults given 50 ppb; there was no difference in response among the groups following the second vaccinal dose (Table 2). The second vaccination resulted in a lesser response than the primary vaccination. The antibody titer response following the first inoculation with SRBC was similar in all poults regardless of diet, but the response following the second inoculation was significantly ($P < 0.05$; Analysis of Variance) lower in poults given dietary aflatoxin than in controls (Table 3).

Table 4 shows liver-to-body-weight ratios determined from weights on the day of necropsy. Turkeys given 150 ppb dietary aflatoxin for 11 weeks and then withdrawn from that diet for 1 week had slightly larger livers than all other turkeys.

Histopathologic examination of kidney, bursa of Fabricius, and thymus from all poults given aflatoxin revealed a normal histologic appearance. Only minor differences were noted in liver sections in poults given aflatoxin, with beginning bile duct proliferation noted in more poults given 150 ppb aflatoxin than in the group fed 50 ppb aflatoxin. The lesions were not more severe or extensive in the group given 150 ppb. These changes did not result in distortion of liver parenchyma, and hepatocellular necrosis was rare. Withdrawal of the poults from the aflatoxin-contaminated diets resulted in fewer poults with bile duct proliferation, but the difference was not marked.

DISCUSSION

In studies where poults were fed 500 ppb dietary AFB_1 from 24 to 42 days of age, the concentration of free or conjugated aflatoxins in liver tissue (2) was similar to what was found in the present study, determining free aflatoxins, at dietary aflatoxin concentrations of 50 or 150 ppb fed for 13 weeks. Perhaps the length of time the poults were fed these diets affected the residue concentrations of the two aflatoxins quantitated. In the study of Gregory *et al.* (2), no attempt was made to determine the residue concentrations of aflatoxins in kidney or gizzard tissue. In the present study, aflatoxin residues remained in gizzard tissues for up to 2 weeks after withdrawal from contaminated diet, but the reason for such is unexplained. The results of the present study are in agreement with other studies (5,8) in that aflatoxins are cleared quite rapidly from most tissues, but not from gizzard tissue and perhaps kidney tissue.

Antibodies against LPS produced by vaccination of chickens and turkeys with *P. multocida* are predominantly of the IgG class (R. B. Rimler, unpublished). The response to the second vaccination probably was lower than response to the first, because the secondary response involved the same class of antibody.

The finding that antibody titers to SRBC were greater before inoculation in the turkeys given dietary aflatoxin may have resulted from the aflatoxin causing a change in the gut flora, thereby allowing for a selectivity of microorganisms with lipopolysaccharides that are cross-reactive to the polysaccharide on the surface of SRBC. Inter-

Table 3. Geometric mean hemagglutination titer of sheep erythrocytes from turkeys fed two concentrations of dietary aflatoxin and inoculated with sheep erythrocytes.

Dietary aflatoxin	Week 4 (before inoculum dose #1)	Week 6	Week 8 (before inoculum dose #2)	Week 10
Control	3.3	279.2	80.6	345.5
50 ppb	14.9	285.6	85.4	174.2
150 ppb	30.4	289.3	98.2	205.0

Table 4. Liver-to-body-wt. ratios of turkeys given dietary aflatoxin for 11 or 13 weeks or given dietary aflatoxin for 11 weeks and then the control diet for 1 or 2 weeks.

Dietary aflatoxin	11 weeks	13 weeks	Aflatoxin + withdrawal	
			1 week	2 weeks
0	0.15	0.12	—	—
50 ppb	0.12	0.13	0.14	0.11
150 ppb	0.13	0.12	0.16	0.13

estingly, both concentrations of dietary aflatoxin decreased the response of turkeys to the second inoculation compared with the control diet. There were only slight differences among the groups in the average geometric mean titers before the second inoculation, so the amount of antibody remaining in the serum of the poults should not have affected the secondary response.

Pier and Heddleston (4) found that the immune response was reduced in turkeys vaccinated with *P. multocida* and fed AFB_1 as low as 250 ppb.

Giambrone *et al.* (1) noted no reduction in either antibody production or resistance to challenge with either Newcastle disease virus or *P. multocida* in poults given the equivalent of 200 ppb dietary AFB_1. Turkeys dosed similarly with the equivalent of 200 ppb dietary AFB_1 plus AFB_2 did not differ notably from controls in cell-mediated immune response. The poults were given the toxin daily for 5 weeks. In the present study, we fed the poults a diet that was not expected to elicit any clinical response or change in the immune response and then assessed the aflatoxin residues in the tissues. However, our data indicate that even these low concentrations of dietary aflatoxins can still elicit a measurable response in poults if they are fed the diets for at least 11 to 13 weeks.

The increase in liver-to-body-weight ratio noted in the turkeys 1 week after withdrawal from the diets containing aflatoxin may be due to increased production of glycogen or other metabolites in the liver. The time period may not have been sufficient for these metabolites to be translocated to other storage depots.

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